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Human Immunodeficiency Virus Types 1 and 2 and Simian Immunodeficiency Virus *env* Proteins Possess a Functionally Conserved Assembly Domain

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The envelope (*env*) glycoproteins of human immunodeficiency viruses type 1 (HIV-1) and type 2 (HIV-2) form dimers shortly after synthesis. Analysis of the simian immunodeficiency virus (SIV) *env* protein expressed by a recombinant vaccinia virus revealed that it, too, forms stable homodimers. When the HIV-1 and SIV *env* proteins or the HIV-1 and HIV-2 *env* proteins were coexpressed in the same cells, heterodimers were formed. Thus, the *env* proteins of HIV-1, HIV-2, and SIV possess a functionally conserved domain involved in subunit-subunit recognition and assembly that likely involves the ectodomain of gp41.

Extreme sequence heterogeneity is a hallmark of the envelope (*env*) proteins of the human immunodeficiency viruses (HIVs) (3, 30). Like the membrane proteins of other viruses, however, *env* proteins must provide conserved functions, which include binding virus to the cell surface and triggering fusion between the viral envelope and a cellular membrane (11, 28). Thus, sequence comparisons between different HIV type 1 (HIV-1) isolates as well as among HIV-1 and the related but distinct HIV-2 and simian immunodeficiency virus (SIV) *env* proteins reveal conserved domains, notably the CD4 binding site in gp120, the cleavage site between gp120 and gp41, and the amino-terminal region of gp41, which is involved in the fusion reaction (1, 14, 15, 17, 27, 31). The ectodomain of gp41 is also highly conserved, which suggests an important role for this region (27). Recently, we have implicated the first 129 amino acids of the HIV-1 gp41 ectodomain as being of critical importance in the assembly of *env* protein oligomers (6). The *env* protein forms noncovalently associated homodimers in the endoplasmic reticulum (ER) which in turn assemble into tetramers (6, 21, 25). Since the *env* proteins are initially synthesized as monomers, newly made subunits must possess the means to recognize each other as appropriate assembly partners in the ER. To further examine the molecular basis of subunit-subunit interactions and to determine whether the assembly site is conserved, we asked whether expression of different primate immunodeficiency viral *env* proteins in the same cell leads to heterologous dimer formation. Such a result would indicate the presence of a structurally and functionally conserved assembly domain.

Earlier electrophoretic studies indicated that the HIV-2 and SIV *env* proteins form sodium dodecyl sulfate (SDS)-resistant dimers transiently during their biosynthesis (23). The existence of HIV-2 gp160 dimers and higher-order oligomeric forms was demonstrated by combined sucrose velocity gradient sedimentation and chemical cross-linking studies (S. Chakrabarti, T. Mizukami, F. Franchini, and B. Moss, Virology, in press). Before the abilities of the various *env* proteins to form hybrid dimers were examined, it was necessary to confirm the oligomeric structure of the SIV *env* protein as well. For this purpose, a recombinant vaccinia virus was used to express the SIV *env* protein in BSC-1 cells (13). Infected cells were lysed in Triton X-100, and the lysate was subjected to sucrose gradient sedimentation. The distribution of *env* protein across the gradient was determined by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting (immunoblotting) with a polyclonal antibody to SIV. Most of the SIV *env* protein was recovered in a peak with a sedimentation coefficient of approximately 12 (Fig. 1). In addition to the 12S material, a smaller amount of protein was recovered in a 8S peak. These $s_{20,w}$ values correspond well to those previously reported for monomeric (7.2S) and dimeric (10.8S) HIV-1 *env* protein (6). Chemical cross-linking confirmed the oligomeric structures of the 8 and 12S proteins. When gradient fractions were cross-linked prior to SDS-PAGE, the 8S material remained monomeric while the 12S material was cross-linked into a band with a molecular mass of approximately 300 to 400 kilodaltons. In addition, a small amount of SIV *env* protein which sedimented at greater than 12S was cross-linked into a higher-order form, probably a tetramer (data not shown). These results were similar to those obtained previously with the HIV-1 *env* protein (6).

Corresponding results were obtained when pulse-chase experiments were performed. Cells expressing the SIV *env* protein were pulse-labeled with [^{35}S]methionine for 20 min and then immediately lysed or chased in the presence of excess cold methionine for 2 h. When the lysates obtained immediately following the pulse-labeling were subjected to sucrose gradient centrifugation, most of the SIV *env* protein sedimented at 8S. After the 2-h chase, however, most of the material sedimented at 12S. Thus, dimers were formed posttranslationally with high efficiency. Dimers also formed in the presence of Brefeldin A, a compound which efficiently and reversibly blocks protein transport from the ER (5, 16), suggesting that assembly occurs in this subcellular compartment (P. Earl, R. Doms, and B. Moss, unpublished data).

To determine if the HIV-1, HIV-2, and SIV *env* proteins could form mixed oligomers, we infected cells with pairs of recombinant vaccinia viruses. The viruses were titrated so that each produced nearly equivalent amounts of *env* protein during double infections, which are easily obtained with vaccinia virus. A coimmunoprecipitation assay was devised to detect mixed oligomers. Lysates from cells expressing

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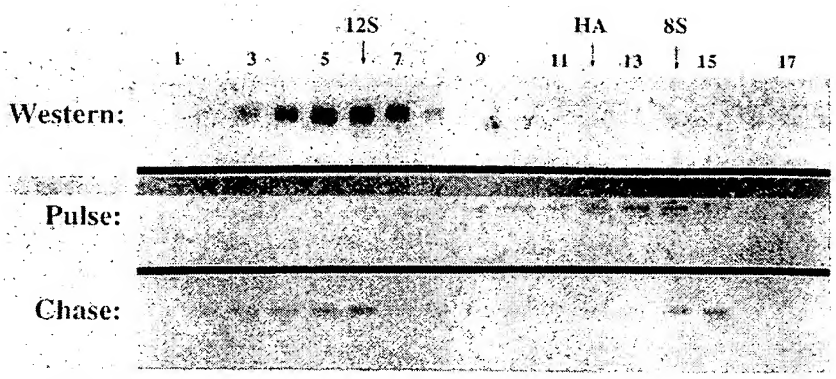

FIG. 1. Sucrose gradient analysis of SIV *env* protein. Confluent BSC-1 cells were infected with a recombinant vaccinia virus (WR194) encoding the SIV *env* protein at a multiplicity of infection of 30 (13). Twenty hours after infection, the cells were lysed with Triton X-100, and the lysate was subjected to velocity gradient sedimentation on a 5 to 20% sucrose gradient at 4°C for 23 h at 40,000 rpm in an SW40 rotor (6). Protein in the gradient fractions was concentrated by trichloroacetic acid precipitation, and the distribution of SIV *env* protein was determined by SDS-PAGE and Western blotting with a polyclonal antibody to SIV (kindly provided by P. Johnson, Georgetown University). Sedimentation coefficients were calculated by standard techniques, as previously described (6). The bottom of the gradient is indicated by fraction 1; the top of the gradient is indicated by fraction 17. Assembly of the SIV *env* protein into dimers was also followed by pulse-chase experiments. BSC-1 cells infected with WR194 were labeled with [^{35}S]methionine (Amersham Corp.) for 20 min at 5 h postinfection. The cells were then immediately lysed (Pulse) or chased in the presence of excess cold methionine for 2 h (Chase). Following centrifugation, the distribution of *env* protein across the gradient was determined by immunoprecipitation with the polyclonal antibody to the SIV *env* protein. HA, Hemagglutinin.

two different *env* proteins were immunoprecipitated with antisera specific for only one of the proteins. Following SDS-PAGE, the material precipitated by the first antibody was transferred to nitrocellulose and probed with an antibody specific for the second *env* protein. A positive result on the Western blot would be expected only if the first antibody coimmunoprecipitated the *env* protein to which it does not directly bind. Two antibodies were used: a rabbit polyclonal serum to HIV-1 gp120 (PB33) and a monkey antiserum to SIV. The SIV serum cross-reacted with the closely related *env* protein of HIV-2 but did not recognize the HIV-1 *env* protein by immunoprecipitation or Western blotting (Fig. 2). Likewise, the antibody to HIV-1 did not recognize either HIV-2 or SIV *env* proteins (Fig. 2).

When cells expressing both HIV-1 and HIV-2 *env* proteins or HIV-1 and SIV *env* proteins were lysed and subjected to the coimmunoprecipitation assay, mixed oligomers were obtained. Antibody to HIV-1 coimmunoprecipitated the HIV-2 and SIV *env* proteins, while the SIV antibody coprecipitated the HIV-1 *env* protein (Fig. 2). To rule out the possibility that coprecipitation was a result of nonspecific interactions arising after lysis of the cells, lysates of singly infected cells were mixed and incubated together for 30 min prior to immunoprecipitation. Coimmunoprecipitation was not observed under these conditions, demonstrating that coprecipitation was specific for cells in which different *env* proteins were coexpressed (Fig. 2). Thus, we concluded that the *env* protein of HIV-1 formed mixed oligomers with the *env* proteins of HIV-2 and SIV when coexpressed in the same cell.

The formation of mixed *env* protein oligomers could be due to formation of correctly folded functional dimers or misfolded aggregates. To differentiate between these possibilities, lysates from double-infected cells were subjected to sucrose velocity gradient sedimentation to separate dimeric *env* protein from monomeric and aggregated forms. We

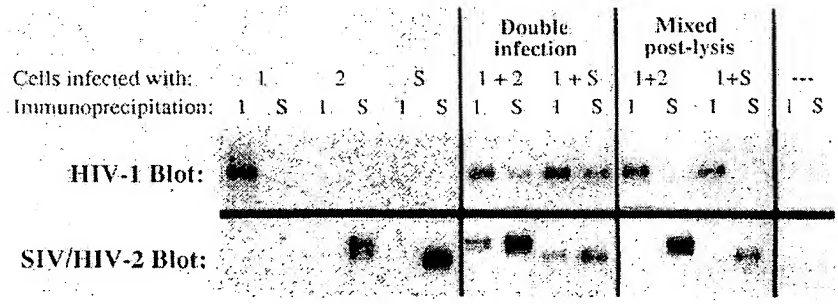

FIG. 2. Formation of mixed HIV-1, HIV-2, and SIV *env* protein oligomers. Cells were infected with recombinant vaccinia viruses encoding the *env* proteins of HIV-1 (6a), HIV-2 (Chakrabarti et al, in press), or SIV alone or in combination. Sixteen hours postinfection, the cells were lysed in Triton X-100 and samples were immunoprecipitated with a rabbit polyclonal antibody to HIV-1 gp120 or a monkey serum to the SIV *env* protein. Equal portions of the immunoprecipitates were run on two polyacrylamide gels, and the proteins were blotted to nitrocellulose. The blots were then probed with antibodies to HIV-1 gp120 or to SIV as indicated, followed by incubation with [^{125}I]protein A (Amersham). To determine if coprecipitation arose after cell lysis, single-infected cell lysates were mixed and incubated at 37°C for 30 min prior to immunoprecipitation (Mixed post-lysis). 1, HIV-1; 2, HIV-2; S, SIV.

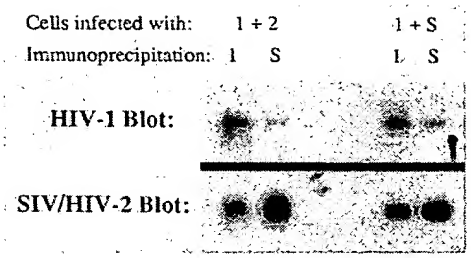

FIG. 3. Formation of heterologous dimers. Lysates of double-infected cells, prepared as for Fig. 2, were subjected to velocity gradient centrifugation, as described in the legend to Fig. 1, to separate monomeric, dimeric, and aggregated forms of the *env* proteins. More than 90% of the *env* protein sedimented in the 11 to 12S region. These fractions were pooled, and samples were precipitated with antibodies to HIV-1 gp120 or SIV *env* protein. The precipitates were then analyzed by SDS-PAGE and Western blotting, as described in the legend to Fig. 2. 1, HIV-1; 2, HIV-2; S, SIV.

found that most of the *env* protein from double-infected cells sedimented in the 11 to 12S region of the gradients, as expected for dimeric *env* protein. Fractions from the 11 to 12S region were pooled, immunoprecipitated, and Western blotted with reciprocal antibodies. Coprecipitation was once again observed (Fig. 3), indicating that the coprecipitation observed directly from the cell lysates (Fig. 2) was due to heterodimer formation. Thus, we concluded that the HIV-1 and HIV-2 as well as the HIV-1 and SIV *env* proteins recognize each other as appropriate assembly partners and form stable, mixed dimers.

The efficiency of heterologous dimer formation was determined by quantitative immunoprecipitations from metabolically labeled cell lysates. By using the approach of Boulay et al. (2), we calculated that approximately 20% of all dimers consisted of heterologous subunits, with the remainder being homodimeric. If all cells are double infected and express equivalent amounts of the two *env* proteins and if mixing of subunits in the ER is random, then up to 50% of the dimers should be mixed (2). The relatively low level of heterodimer formation observed was most likely due to preferential association between subunits arising from the same polysome or by some degree of preferential association between homologous subunits rather than to unequal levels of expression or instability of mixed dimers to immunoprecipitation. Additional experiments are required to distinguish between these possibilities.

The ability to efficiently coexpress proteins with recombinant vaccinia viruses offers the means of studying subunit binding sites by investigating whether subunits from different species or strains can form mixed oligomers. In the case of the trimeric influenza hemagglutinin, subunit-subunit recognition requires a high degree of structural complementarity between subunits (2, 26). While hemagglutinins from different strains generally form mixed trimers, those from different subtypes usually do not. In the latter case, the subunits typically have less than 50% sequence identity. Given this, it was somewhat surprising that the HIV-1 *env* protein formed mixed dimers with the *env* proteins from HIV-2 and SIV, with which it has less than 40% amino acid identity. Does heterologous dimer formation indicate the presence of a highly conserved structural domain? We have shown that the 129 amino-terminal residues of the gp41 ectodomain are both necessary and sufficient for dimerization of the HIV-1 *env* protein (6). The gp41 subunits of the

TABLE 1. *env* protein sequence comparisons

Viruses	% Amino acid identity[a]				
	gp160	gp120	gp41	gp41 cytoplasmic domain	gp41 ectodomain
HIV-1, HIV-2	35	31	40	25	55
HIV-1, SIV	35	30	41	31	56
HIV-2, SIV	69	69	68	53	81

[a] The *env* protein sequences of HIV-1_{BH8} (22), HIV-$2_{SBL6669}$ (8), and SIV_{Mac} (7) were aligned, and the amino acid identity percentages were determined as described by Pearson and Lipman (20). The gp41 ectodomain was defined as the amino-terminal 129 amino acids of gp41, which have been shown to be both necessary and sufficient for *env* protein oligomerization (6). The cytoplasmic domain was defined as all residues C terminal to amino acid 195 in gp41 (10).

HIV-1, HIV-2, and SIV *env* proteins are more highly conserved than their gp120 counterparts (Table 1). Within gp41, the ectodomain is more highly conserved than either the cytoplasmic or transmembrane domains (Table 1). Given this degree of conservation and our studies on the HIV-1 *env* protein (6), it is likely that the ectodomain region of the membrane-spanning subunit in all three immunodeficiency virus *env* proteins is largely responsible for subunit-subunit recognition and assembly.

The presence of a conserved assembly site in the gp41 ectodomain may have important implications. By analogy with other viral and cellular membrane proteins, assembly into dimers and tetramers is likely to be a prerequisite for transport from the ER and for incorporation into virus (12, 24). This requirement, manifested as a conserved assembly domain in gp41, is likely to place severe constraints on amino acid substitutions in this region. Studies with influenza hemagglutinin, for example, show that even single amino acid substitutions can drastically affect oligomer stability (2, 4). Conservation of the *env* protein assembly domain has been confirmed immunologically, in that antibodies to this region exhibit a strong tendency to cross-react with different virus isolates (9, 18, 19, 29). Interestingly, some of these antibodies fail to react with native protein (19). Whether residues making up these epitopes participate directly in subunit-subunit interactions and so are inaccessible in the subunit interface region needs to be examined.

In summary, the gp41 assembly domain joins those involved in CD4 binding, gp120-gp41 subunit cleavage, and membrane fusion as representing regions of functional importance which share, as a result, a considerable degree of structural similarity. The identification of these and other conserved sites will undoubtably lead to a greater understanding of the structure and function of the molecule and may suggest regions to which cross-reactive neutralizing antibodies can be directed. Finally, heterologous dimers formed from coexpressed wild-type and mutant *env* protein molecules can be assayed for their abilities to form dimers, bind CD4, be incorporated into virus, and trigger membrane fusion, which offers an interesting way to study the role of subunit cooperativity in *env* protein function.

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